

CLAIMS

We claim:

- 5    1.    A chemiluminescent substrate of a hydrolytic enzyme  
having the structure

Lumi-M-P

10    wherein:

- a.    Lumi is a chemiluminescent moiety,  
         b.    M is a multivalent heteroatom, having at least  
         one lone pair electrons, directly attached to said  
         Lumi and to P, and  
15    c.    P is a group that can be removed by hydrolytic  
         enzymes.

2.    The chemiluminescent substrate of claim 1 wherein:

         Lumi is selected from the group consisting of  
20    acridinium compounds, benzacridium compounds, quinolinium  
         compounds, isoquinilinium compounds, phenanthridium  
         compounds, lucigenin compounds, acridans or other reduced  
         forms of the above, acridines or other non-N-alkylated  
         forms of the above, spiroacridan compounds, luminol  
25    compounds and isoluminol compounds; and

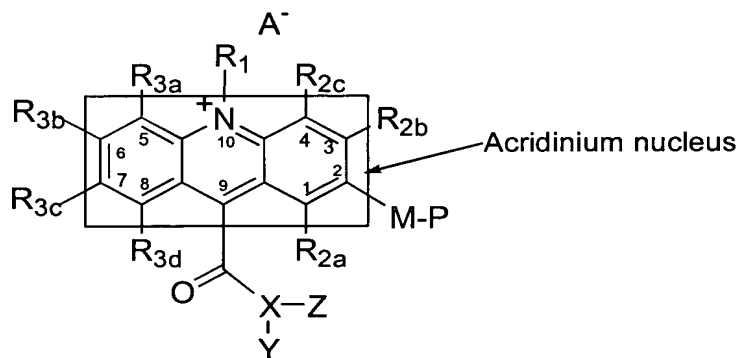
         M is selected from the group consisting of oxygen,  
nitrogen and sulfur.

3. The chemiluminescent substrate of claim 1 wherein

M is a multivalent heteroatom selected from the group consisting of oxygen, nitrogen and sulfur; and

P is a group that is thermally and hydrolytically stable in aqueous medium and is removable by a hydrolytic enzyme.

4. The chemiluminescent substrate of claim 2 wherein said Lumi chemiluminescent moiety is an acridinium compound having the following structure:



wherein:

P is a group that is thermally and hydrolytically stable in aqueous medium and readily removable by a hydrolytic enzyme to form Lumi-M;

M is oxygen, nitrogen or sulfur;

R<sub>1</sub> is an alkyl, alkenyl, alkynyl or aralkyl containing 0 to 20 heteroatoms;

A<sup>-</sup> is a counter ion for the electroneutrality of the quaternary nitrogen of the acridinium compounds, said A<sup>-</sup> not being present if said R<sub>1</sub> substituent contains a strongly ionizable group that can form an anion and pair with the quaternary ammonium cationic moiety;

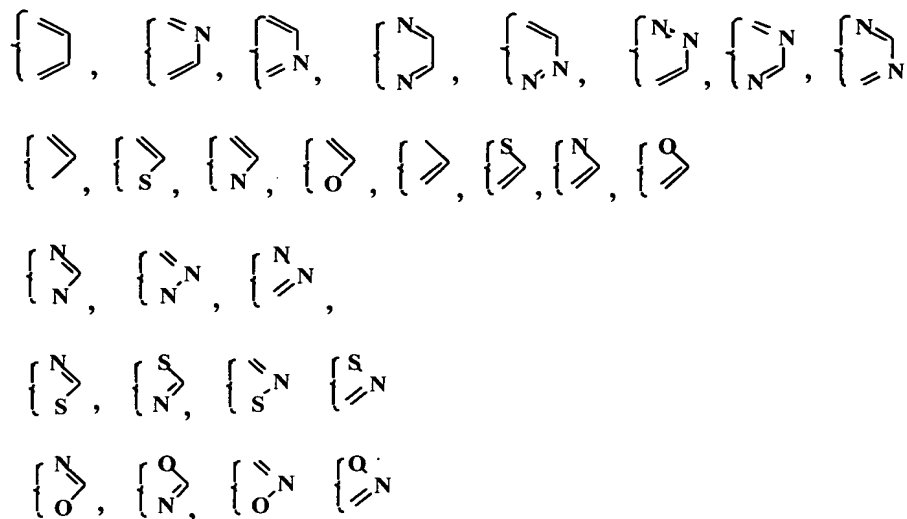
when X is oxygen, Z is omitted and Y is a substituted or unsubstituted aryl group or  $-N=CR_9R_{10}$ ,  
20 wherein  $R_9$  and  $R_{10}$  may be the same or different and are selected from hydrogen, substituted or non-substituted aryl, alkyl, alkenyl, alkynyl, halide, alkoxy and aryloxy groups;

when X is nitrogen, Z is  $-SO_2-Y'$ , Y' being defined the same as Y above; Y is as defined above or can be a branched or straight-chain alkyl containing 0 to 20 carbons, halogenated or

5 5. The chemiluminescent substrate of claim 4 wherein R<sub>1</sub> is methyl, sulfoalkyl or an alkyl containing one or more hydrophilic groups selected from the group consisting of sulfonate, sulfate, -CO<sub>2</sub>H, phosphonate, ethylene glycol, polyethylene glycol, quaternary ammonium (-N<sup>+</sup>R<sub>3</sub>), and any  
10 groups containing one or more of said hydrophilic groups.

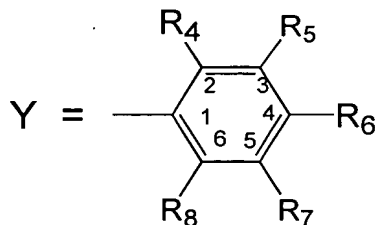
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7. The chemiluminescent substrate of claim 4 wherein any two adjacent substituents at the acridinium nucleus positions can be linked to form additional carbocyclic and heterocyclic rings fused to the attached acridinium nucleus, said rings being selected from the group consisting of:



8. The chemiluminescent substrate of claim 4 wherein said counter ions A are selected from the group consisting of  
 5  $\text{CH}_3\text{SO}_4^-$ ,  $\text{FSO}_3^-$ ,  $\text{CF}_3\text{SO}_3^-$ ,  $\text{C}_4\text{F}_9\text{SO}_3^-$ ,  $\text{CH}_3\text{C}_6\text{H}_4\text{SO}_3^-$ , halide,  $\text{CF}_3\text{COO}^-$ ,  $\text{CH}_3\text{COO}^-$ , and  $\text{NO}_3^-$ .

9. The chemiluminescent substrate of claim 4 wherein X is oxygen or sulfur, Z is omitted, Y is a polysubstituted aryl group of the following formula:  
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where  $R_4$  and  $R_8$  can be the same or different and are alkyl, alkenyl, alkynyl, alkoxyl (-OR), alkylthiol (-SR), or substituted amino groups.

5 10. The chemiluminescent substrate of claim 9 wherein  $R_5$ ,  $R_6$  and  $R_7$  are the same or different, and are hydrogen, -R, substituted or unsubstituted aryl, halides, amino, -NHR, -NR<sub>2</sub>, quaternary ammonium (-N<sup>+</sup>R<sub>3</sub>), hydroxyl, nitro, nitroso, sulfonate, sulfate, cyano (-CN), phosphonate,  
10 CO<sub>2</sub>H, -SCN, -OR, -SR, -SSR, -C(O)R, -C(O)NHR, -NHC(O)R, ethylene glycol, or polyethyelene glycol.

11. The chemiluminescent substrate of claim 9 wherein  $R_4$  and  $R_8$  are short chain alkyl groups containing 1-10 carbons,  
15 preferably methyl groups, or at least one of  $R_4$  and  $R_8$  is as defined while the other is a hydrogen or a halides.

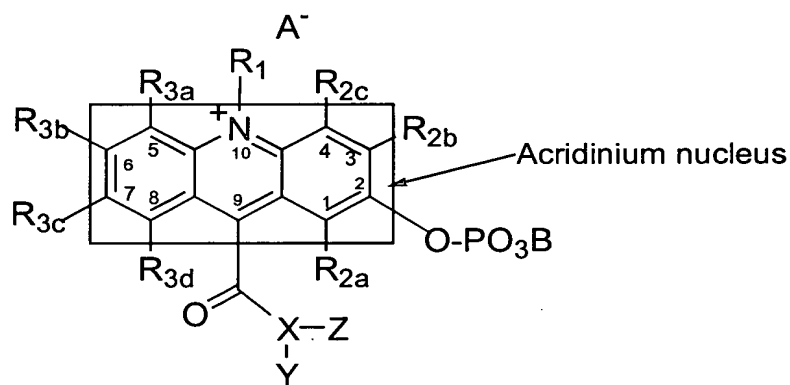
12. The chemiluminescent substrate of claim 9 wherein any adjacent two groups of said  $R_4$  to  $R_8$  can form one or more  
20 additional fused hydrocarbon aromatic rings or heteroaromatic rings with or without substitutions, selected from the group consisting of benzene, naphthlene, pyridine, thiophene, furan, and pyrrole.

25 13. The chemiluminescent substrate of claim 9 wherein  $R_5$ ,  $R_6$  and  $R_7$  can be the same or different and comprise hydrophilic groups selected from the group consisting of sulfonate, sulfate, -CO<sub>2</sub>H, phosphonate, ethylene glycol, polyethylene glycol, quaternary ammonium (-N<sup>+</sup>R<sub>3</sub>), and any  
30 groups containing one or more of said hydrophilic groups.

14. The chemiluminescent substrate of claim 4 wherein after the removal of P by a hydrolytic enzyme, M becomes ionizable in the medium of the reaction to bear a negative charge, thus strongly donating electrons to the acridinium ring system.

15. The chemiluminescent substrate of claim 4 having the following structure:

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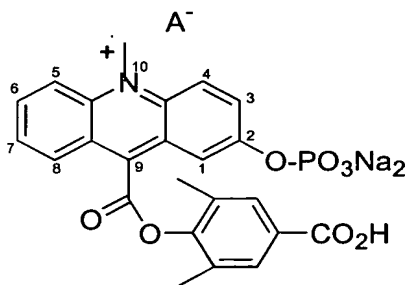
wherein B is either a divalent cation or two monovalent cations, said monovalent cations being the same or different.

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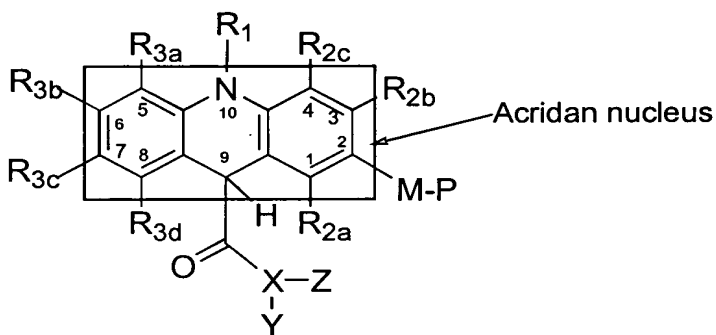
16. The chemiluminescent substrate of claim 15 wherein, if B is a monovalent cation, each B is selected from the group consisting of sodium, hydrogen, potassium, ammonium, and, or, if B is a divalent cation, B is calcium and magnesium.

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17. The chemiluminescent substrate of claim 16 having the following structure:



18. The chemiluminescent substrate of claim 2 wherein said chemiluminescent moiety Lumi is an acridan compound having the following structure:



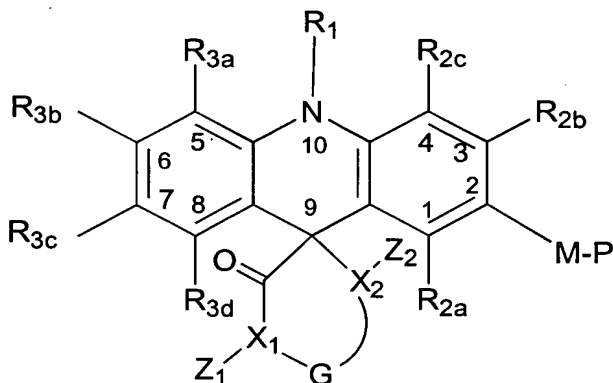
wherein,  $R_1$ ,  $R_{2a-c}$ ,  $R_{3a-d}$ , M, P, X, Y, and Z are as defined in claim 4.

19. The chemiluminescent substrate of claim 18, wherein M-P is  $O-PO_3Na_2$ .

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20. The chemiluminescent substrate of claim 2 wherein said chemiluminescent moiety Lumi is a [n] spiroacridan compound, having the following structure:



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wherein,  $R_1$ ,  $R_{2a-c}$ ,  $R_{3a-d}$ , M and P are as defined in claim 4;

$X_1$  and  $X_2$  are the same or different and are selected from the group consisting of oxygen, sulfur and nitrogen, and when either one or both of  $X_1$  and  $X_2$  are oxygen or sulfur, the corresponding  $Z_1$  or  $Z_2$  or both  $Z_1$  and  $Z_2$  are omitted; when one or both of  $X_1$  or  $X_2$  are nitrogen, the corresponding  $Z_1$  or  $Z_2$  or both  $Z_1$  and  $Z_2$  are hydrogen, alkyl, aryl or  $-SO_2-Y'$ ; and

G is a group connecting  $X_1$  and  $X_2$  to form a ring having 5 to 10 members.

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